AMENDMENT TO THE SPECIFICATION

Please replace the last paragraph of page 93 (paragraph [0337] of the published application) with the following rewritten paragraph:

In the context of the adzyme linker design, once the address and the catalytic domain is chosen, molecular model of the target – adzyme avid complex may be obtained. d0, the distance between the point where the linker connects to the address and the point where the linker connects to the enzyme, while both the address and enzyme domain are in the avid complex, can be readily determined from, for example, the 3-D structure of the target – adzyme complex. Many cytokine structures are solved (see the Cytokine Web site at cmbi.bjmu dot edu.cn/cmbidata/cgf/CGF_Database/cytweb/cyt_strucs/index.html ~~http://cmbi.bjmu.edu.cn/cmbidata/cgf/CGF_Database/cytweb/cyt_strucs/index.html~~). The structure of those other cytokines with sequence homology to cytokines of known structures, as well as the target – adzyme complex may be routinely obtained via molecular modeling.

Please replace the last full paragraph of page 151 (paragraph [0574] of the published application) with the following rewritten paragraph:

a. Selection of the enzyme domains A survey of the literature and public domain databases (MEROPS: merops.sanger.ac dot uk ~~http://www.merops.sanger.ac.uk~~) for proteases that are commercially available, expressible as zymogens, and expected to cleave and inactivate TNFα [19-24] led to the selection of twenty candidate proteases, which were then tested for inactivation of TNFα using a TNF cytotoxicity assay. Specifically, TNF activation of functional TNFα receptor TNFR-1 [10, 25] leads to apoptotic cell death, which can be quantified in a cell-based assay [26]. This assay served as the basis to screen the 20 proteases for inactivation of TNFα bioactivity (see below, Fig. 10, Table 2).

Please replace the paragraph bridging pages 154 and 155 (paragraph [0584] of the published application) with the following rewritten paragraph:

As an alternative to using sp55 as an address domain, one anti-TNFα scFV antibody will be selected from a set of eighteen that were obtained from Genetastix (San Jose, CA). These scFV antibodies were identified by Genetastix through use of their proprietary technology (genetastix dot com ~~www.genetastix.com~~) as having TNFα binding activity. Briefly, a human scFv cDNA library was produced from polyA RNA of human spleen, lymph nodes and peripheral blood lymphocytes through amplification of V_H and V_L sequences that were assembled in frame with a GAL4 activation domain (AD). The 18 scFvs were identified as binding human TNFα-lexA DNA binding domain when co-expressed intracellularly in yeast. The Genetastix scFvs expression vectors were obtained in the form of bacterial periplasmic expression vector pET25B (Novagen, Madison, WI). Standard recombinant DNA methods were used to subclone the scFv coding sequences into the pSecTag2A vector. The constructs were then sequenced to verify the structures. These scFv anti-TNFα antibodies is expressed and purified as described for the previous adzyme components, then analyzed for binding to TNFα. An indirect ELISA is used for TNFα based on the S-Tag™ system (see above, Fig. 11) to identify one of the 18 scFvs that shows high affinity binding to TNFα for use as an address domain. The selection of a specific scFv is based on a ranking of their relative binding strengths of the various structures. Further quantitative determinations of binding affinities for TNFα may be included once a prototype adzyme has been identified.

Please delete the paragraph on page 2, line 26 to page 3, line 3 and replace it with the following paragraph:

In certain embodiments, a catalytic domain and a targeting domain of the adzyme are joined by a polypeptide linker to form a fusion protein. A fusion protein may be generated in a variety of ways, including chemical coupling and cotranslation. In a preferred embodiment, the fusion protein is a cotranslational fusion protein encoded by a recombinant nucleic acid. In

certain embodiments the linker for the fusion protein is an unstructured peptide. Optionally, the linker includes one or more repeats of Ser_4Gly (SEQ ID NO: 7) or $SerGly_4$ (SEQ ID NO: 8). In preferred embodiments, the linker is selected to provide steric geometry between said catalytic domain and said targeting moiety such that said adzyme is more effective against the substrate than either the catalytic domain or targeting moiety alone. For example, the linker may be selected such that the adzyme is more potent than said catalytic domain or targeting moiety with respect to the reaction with said substrate. The linker may be selected such that the targeting moiety presents the substrate to the enzymatic domain at an effective concentration at least 5 fold greater than would be present in the absence of the targeting moiety.

Please delete the paragraph on page 6, line 27 to page 7, line 3 and replace it with the following paragraph:

In certain embodiments of an adzyme having one or more of such properties with respect to the reaction with the substrate molecule, a catalytic domain and a targeting domain of the adzyme are joined by a polypeptide and linker to form a fusion protein. The fusion protein may be generated in a variety of ways, including chemical coupling and cotranslation. In a preferred embodiment, the fusion protein is a cotranslational fusion protein encoded by a recombinant nucleic acid. In certain embodiments the linker for the fusion protein is an unstructured peptide. Optionally, the linker includes one or more repeats of Ser_4Gly (SEQ ID NO: 7) or $SerGly_4$ (SEQ ID NO: 8). In preferred embodiments, the linker is selected to provide steric geometry between said catalytic domain and said targeting moiety such that said adzyme is more effective against the substrate than either the catalytic domain or targeting moiety alone. For example, the linker may be selected such that the adzyme is more potent than said catalytic domain or targeting moiety with respect to the reaction with said substrate. The linker may be selected such that the targeting moiety presents the substrate to the enzymatic domain at an effective concentration at least 5 fold greater than would be present in the absence of the targeting moiety.

Please delete the paragraph on page 9, line 33 to page 10, line 10 and replace it with the following paragraph:

In certain embodiments of an adzyme that targets an extracellular signaling molecule, a catalytic domain and a targeting domain of the adzyme are joined by a polypeptide and linker to form a fusion protein. A fusion protein may be generated in a variety of ways, including chemical coupling and cotranslation. In a preferred embodiment, the fusion protein is a cotranslational fusion protein encoded by a recombinant nucleic acid. In certain embodiments the linker for the fusion protein is an unstructured peptide. Optionally, the linker includes one or more repeats of Ser_4Gly (SEQ ID NO: 7) or $SerGly_4$ (SEQ ID NO: 8). In preferred embodiments, the linker is selected to provide steric geometry between said catalytic domain and said targeting moiety such that said adzyme is more effective against the substrate than either the catalytic domain or targeting moiety alone. For example, the linker may be selected such that the adzyme is more potent than said catalytic domain or targeting moiety with respect to the reaction with said substrate. The linker may be selected such that the targeting moiety presents the substrate to the enzymatic domain at an effective concentration at least 5 fold greater than would be present in the absence of the targeting moiety.

Please delete the paragraph on page 13, lines 11-25 and replace it with the following paragraph:

In certain embodiments of an adzyme that targets a receptor, a catalytic domain and a targeting domain of the adzyme are joined by a polypeptide and linker to form a fusion protein. A fusion protein may be generated in a variety of ways, including chemical coupling and cotranslation. In a preferred embodiment, the fusion protein is a cotranslational fusion protein encoded by a recombinant nucleic acid. In certain embodiments the linker for the fusion protein is an unstructured peptide. Optionally, the linker includes one or more repeats of Ser_4Gly (SEQ ID NO: 7) or $SerGly_4$ (SEQ ID NO: 8). In preferred embodiments, the linker is selected to provide steric geometry between said catalytic domain and said targeting moiety such that said adzyme is more effective against the substrate than either the catalytic domain or targeting moiety alone. For example, the linker may be selected such that the adzyme is more potent than

said catalytic domain or targeting moiety with respect to the reaction with said substrate. The linker may be selected such that the targeting moiety presents the substrate to the enzymatic domain at an effective concentration at least 5 fold greater than would be present in the absence of the targeting moiety.

Please delete the paragraph on page 16, lines 7-21 and replace it with the following paragraph:

In certain embodiments of an adzyme that generates an antagonist of the substrate, a catalytic domain and a targeting domain of the adzyme are joined by a polypeptide and linker to form a fusion protein. A fusion protein may be generated in a variety of ways, including chemical coupling and cotranslation. In a preferred embodiment, the fusion protein is a cotranslational fusion protein encoded by a recombinant nucleic acid. In certain embodiments the linker for the fusion protein is an unstructured peptide. Optionally, the linker includes one or more repeats of Ser_4Gly (SEQ ID NO: 7) or $SerGly_4$ (SEQ ID NO: 8). In preferred embodiments, the linker is selected to provide steric geometry between said catalytic domain and said targeting moiety such that said adzyme is more effective against the substrate than either the catalytic domain or targeting moiety alone. For example, the linker may be selected such that the adzyme is more potent than said catalytic domain or targeting moiety with respect to the reaction with said substrate. The linker may be selected such that the targeting moiety presents the substrate to the enzymatic domain at an effective concentration at least 5 fold greater than would be present in the absence of the targeting moiety.

Please delete the paragraph on page 19, line 29 to page 20, line 5 and replace it with the following paragraph:

In certain embodiments of a proteolytic adzyme, a catalytic domain and a targeting domain of the adzyme are joined by a polypeptide and linker to form a fusion protein. A fusion protein may be generated in a variety of ways, including chemical coupling and cotranslation. In a preferred embodiment, the fusion protein is a cotranslational fusion protein encoded by a

recombinant nucleic acid. In certain embodiments the linker for the fusion protein is an unstructured peptide. Optionally, the linker includes one or more repeats of Ser_4Gly (SEQ ID NO: 7) or $SerGly_4$ (SEQ ID NO: 8). In preferred embodiments, the linker is selected to provide steric geometry between said catalytic domain and said targeting moiety such that said adzyme is more effective against the substrate than either the catalytic domain or targeting moiety alone. For example, the linker may be selected such that the adzyme is more potent than said catalytic domain or targeting moiety with respect to the reaction with said substrate. The linker may be selected such that the targeting moiety presents the substrate to the enzymatic domain at an effective concentration at least 5 fold greater than would be present in the absence of the targeting moiety.

Please delete the paragraph on page 22, line 30 to page 24, line 23 and replace it with the following paragraph:

In certain aspects, the invention provides an adzyme for inhibiting receptor-mediated signaling activity of an extracellular substrate polypeptide, the adzyme being a fusion protein comprising a protease domain that catalyzes the proteolytic cleavage of at least one peptide bond of the substrate polypeptide so as to inhibit the receptor-mediated signaling activity of the polypeptide, and a targeting domain that reversibly binds with an address site on said substrate polypeptide, wherein said targeting domain and said protease domain are discrete and heterologous with respect to each other. Optionally, the adzyme is resistant to cleavage by said protease domain. Optionally, the protease domain is a zymogen. Optionally, the protease domain is selected from among: a serine proteinase, a cysteine protease, a threonine protease, an aspartate protease and a metalloproteinase. Optionally, the adzyme is purified from a cell culture in the presence of a reversible protease inhibitor that inhibits the protease activity of the protease domain. In certain embodiments, the adzyme has one or more properties, with respect to the reaction with said substrate In many instances, such properties will be significant for achieving the desired effect of the adzyme on the substrate. For example, an adzyme may have a potency at least 2 times greater than the catalytic domain or the targeting moiety alone, and preferably at

least 3, 5, 10, 20 or more times greater than the potency of the catalytic domain or targeting moiety alone. The adzyme may have a k_{on} of 10^3 $M^{-1}s^{-1}$ or greater, and optionally a k_{on} of 10^4 $M^{-1}s^{-1}$, 10^5 $M^{-1}s^{-1}$, 10^6 $M^{-1}s^{-1}$, 10^7 $M^{-1}s^{-1}$ or greater. The adzyme may have a k_{cat} of 0.1 sec^{-1} or greater, and optionally a k_{cat} of 1 sec^{-1}, 10 sec^{-1}, 50 sec^{-1} or greater. The adzyme may have a K_D that is at least 5, 10, 25, 50 or 100 or more fold less than the K_m of the catalytic domain. The adzyme may have a k_{off} of 10^{-4} sec^{-1} or greater, and optionally a k_{off} of 10^{-2} sec^{-1}, k_{off} of 10^{-2} sec^{-1}, or greater. The adzyme may have a catalytic efficiency that is at least 5 fold greater than the catalytic efficiency of the catalytic domain alone, and optionally a catalytic efficiency that is at least 10 fold, 20 fold, 50 fold or 100 fold greater than that of the catalytic domain. The adzyme may have a K_m at least 5 fold, 10 fold, 20 fold, 50 fold, or 100 fold less than the K_m of the catalytic domain alone. The adzyme may have an effective substrate concentration that is at least 5 fold, 10 fold, 20 fold, 50 fold or 100 fold greater than the actual substrate concentration. In certain preferred embodiments, an adzyme will be designed so as to combine two or more of the above described properties. Optionally, the substrate is an inflammatory cytokine. In a preferred embodiment, the substrate is an interleukin-1 (e.g., IL-1α, IL-1β) or a TNF- α. In certain embodiments, the substrate is a polypeptide hormone, a growth factor and/or a cytokine, especially an inflammatory cytokine. Optionally, the adzyme acts to reduces a pro-inflammatory activity of a substrate. A substrate may be selected from is selected from the group consisting of four-helix bundle factors, EGF-like factors, insulin-like factors, β-trefoil factors and cysteine knot factors. In a preferred embodiment, the substrate is endogenous to a human patient. In such an embodiment, the adzyme is preferably effective against the substrate in the presence of physiological levels of an abundant human serum protein, such as, serum albumins or an abundant globin. The fusion protein adzymes may be generated in a variety of ways, including chemical coupling and cotranslation. In a preferred embodiment, the fusion protein is a cotranslational fusion protein encoded by a recombinant nucleic acid. In certain embodiments the linker for the fusion protein is an unstructured peptide. Optionally, the linker includes one or more repeats of Ser_4Gly (SEQ ID NO: 7) or $SerGly_4$ (SEQ ID NO: 8). In preferred embodiments, the linker is selected to provide steric geometry between said catalytic domain and said targeting moiety such that said adzyme is more effective against the substrate than either the catalytic domain or targeting moiety alone. For example, the linker may be selected such that the

adzyme is more potent than said catalytic domain or targeting moiety with respect to the reaction with said substrate. The linker may be selected such that the targeting moiety presents the substrate to the enzymatic domain at an effective concentration at least 5 fold greater than would be present in the absence of the targeting moiety. A targeting domain may include essentially any molecule or assembly of molecules that binds to the address site (e.g., on the substrate in the case of direct adzymes or on a molecule that occurs in functional proximity to the substrate, in the case of proximity adzymes). In many embodiments, a targeting domain will comprise an antigen binding site of an antibody, such as a single chain antibody. Optionally, the targeting moiety is an artificial protein or peptide sequence engineered to bind to the substrate. In a preferred embodiment, the substrate for the adzyme is TNFα. In the case of a direct adzyme, the targeting moiety binds to TNFα. Preferably, the catalytic domain comprises a protease that decreases TNFα activity. For example, the protease is may be selected from among: MT1-MMP; MMP12; tryptase; MT2-MMP; elastase; MMP7; chymotrypsin; and trypsin. The targeting moiety may be selected from among, a soluble portion of a TNFα receptor and a single chain antibody that binds to TNFα, although other targeting moieties are possible. A preferred targeting moiety is an sp55 portion of TNFα Receptor 1 (TNFR1). In another preferred embodiment, the substrate for the adzyme is an interleukin-1, such as IL-1α or IL-1β. In the case of a direct adzyme, the targeting moiety binds to the interleukin-1 substrate. Preferably, the catalytic domain comprises a protease that decreases an IL-1 bioactivity.

Please delete the paragraph on page 36, lines 24-36 and replace it with the following paragraph:

The "metalloproteinases" are found in bacteria, fungi as well as in higher organisms. They differ widely in their sequences and their structures but the great majority of enzymes contain a zinc (Zn) atom which is catalytically active. In some cases, zinc may be replaced by another metal such as cobalt or nickel without loss of the activity. Bacterial thermolysin has been well characterized and its crystallographic structure indicates that zinc is bound by two histidines and one glutamic acid. Many enzymes contain the sequence HEXXH (SEQ ID NO: 9), which provides two histidine ligands for the zinc whereas the third ligand is either a glutamic acid

(thermolysin, neprilysin, alanyl aminopeptidase) or a histidine (astacin). Other families exhibit a distinct mode of binding of the Zn atom. The catalytic mechanism leads to the formation of a non-covalent tetrahedral intermediate after the attack of a zinc-bound water molecule on the carbonyl group of the scissile bond. This intermediate is further decomposed by transfer of the glutamic acid proton to the leaving group.

Please delete the paragraph on page 45, lines 8-13 and replace it with the following paragraph:

The fusion partners can, for example, be $(histidine)_6$-tag (SEQ ID NO: 10), glutathione S-transferase, protein A, dihydrofolate reductase, Tag•100 epitope (EETARFQPGYRS; SEQ ID NO:1), c-myc epitope (EQKLISEEDL; SEQ ID NO:2), FLAG®-epitope (DYKDDDK; SEQ ID NO:3), lacZ, CMP (calmodulin-binding peptide), HA epitope (YPYDVPDYA; SEQ ID NO:4), protein C epitope (EDQVDPRLIDGK; SEQ ID NO:5) or VSV epitope (YTDIEMNRLGK; SEQ ID NO:6).

Please delete the paragraph on page 63, lines 14-23 and replace it with the following paragraph:

Another example of an internalizing peptide is the HIV transactivator (TAT) protein. This protein appears to be divided into four domains (Kuppuswamy *et al.* (1989) *Nucl. Acids* Res. 17:3551-3561). Purified TAT protein is taken up by cells in tissue culture (Frankel and Pabo, (1989) *Cell* 55:1189-1193), and peptides, such as the fragment corresponding to residues 37 -62 of TAT, are rapidly taken up by cell *in vitro* (Green and Loewenstein, (1989) *Cell* 55:1179-1188). The highly basic region mediates internalization and targeting of the internalizing moiety to the nucleus (Ruben *et al.*, (1989) *J. Virol.* 63:1-8). Peptides or analogs that include a sequence present in the highly basic region, such as CFITKALGISYGRKKRRQRRRPPQGS (SEQ ID NO: 11), can be used in the adzyme to aid in internalization.

Please delete the paragraph on page 63, line 27 to page 64, line 5 and replace it with the following paragraph:

While not wishing to be bound by any particular theory, it is noted that hydrophilic polypeptides and organic molecules may be also be physiologically transported across the membrane barriers by coupling or conjugating the polypeptide to a transportable peptide which is capable of crossing the membrane by receptor-mediated transcytosis. Suitable internalizing peptides of this type can be generated using all or a portion of, e.g., a histone, insulin, transferrin, basic albumin, prolactin and insulin-like growth factor I (IGF-I), insulin-like growth factor II (IGF-II) or other growth factors. For instance, it has been found that an insulin fragment, showing affinity for the insulin receptor on capillary cells, and being less effective than insulin in blood sugar reduction, is capable of transmembrane transport by receptor-mediated transcytosis and can therefore serve as an internalizing peptide for the subject adzyme. Preferred growth factor-derived internalizing peptides include EGF (epidermal growth factor)-derived peptides, such as CMHIESLDSYTC (SEQ ID NO: 12) and CMYIEALDKYAC (SEQ ID NO: 13); TGF-beta (transforming growth factor beta)-derived peptides; peptides derived from PDGF (platelet-derived growth factor) or PDGF-2; peptides derived from IGF-I (insulin-like growth factor) or IGF-II; and FGF (fibroblast growth factor)-derived peptides.

Please delete the paragraph on page 64, lines 22-32 and replace it with the following paragraph:

A particularly preferred pH-dependent membrane-binding internalizing peptide in this regard is Xaa1-Xaa2-Xaa3-EAALA(EALA)4-EALEALAA-amide (SEQ ID NO: 14), which represents a modification of the peptide sequence of Subbarao *et al.* (*Biochemistry* 26:2964, 1987). Within this peptide sequence, the first amino acid residue (Xaa1) is preferably a unique residue, such as cysteine or lysine, that facilitates chemical conjugation of the internalizing peptide to a targeting protein conjugate. Amino acid residues Xaa2-Xaa3 may be selected to modulate the affinity of the internalizing peptide for different membranes. For instance, if both residues 2 and 3 are lys or arg, the internalizing peptide will have the capacity to bind to

membranes or patches of lipids having a negative surface charge. If residues 2-3 are neutral amino acids, the internalizing peptide will insert into neutral membranes.

Please delete the paragraph on page 74, lines 3-8 to and replace it with the following paragraph:

In still other embodiments, the TNFα targeting moiety is a peptide. For instance, Guo et al. (2002) Di Yi Jun Yi Da Xue Xue Bao. 22(7):597 describes the screening of TNFα-binding peptides by phage display. That reference teaches a number of short peptides that could be used to generate TNFα-targeted adzymes. Merely to illustrate, the TNFα targeting moiety can be a peptide having the sequence ALWHWWH (SEQ ID NO: 15) or (T/S)WLHWWA (SEQ ID NO: 16).

Please delete the paragraph on page 92, lines 14-24 and replace it with the following paragraph:

In some instances it may be necessary to introduce a polypeptide linker region between portions of the chimeric protein derived from different proteins. This linker can facilitate enhanced flexibility of the fusion protein allowing various portions to freely and (optionally) simultaneously interact with a target by reducing steric hindrance between the portions, as well as allowing appropriate folding of each portion to occur. The linker can be of natural origin, such as a sequence determined to exist in random coil between two domains of a protein. Alternatively, the linker can be of synthetic origin. For instance, the sequence $(Gly_4Ser)_n$ (SEQ ID NO: 17) can be used as a synthetic unstructured linker. Linkers of this type are described in Huston et al. (1988) PNAS 85:4879; and U.S. Patent Nos. 5,091,513 and 5,258,498. Naturally occurring unstructured linkers of human origin are preferred as they reduce the risk of immunogenicity.

Please delete the paragraph on page 94, lines 24-28 and replace it with the following paragraph:

Fusion proteins can comprise additional sequences, including a leader (or signal peptide) sequence, a portion of an immunoglobulin (e.g., an Fc portion, see below) or other oligomer-forming sequences, as well as sequences encoding highly antigenic moieties, hexahistidine (SEQ ID NO: 10) moieties or other elements that provide a means for facile purification or rapid detection of a fusion protein.

Please delete the paragraphs on page 141, line 20 to page 143, line 9 and replace them with the following paragraphs:

In Figure 4, all components were assembled in the pSecTag2A vector system (Invitrogen, Carlsbad, CA), which included an N-terminal leader peptide designed to enable secretion from a heterologous expression system and C-terminal tandem myc and His_6 (SEQ ID NO: 10) tags to enable immunodetection and purification. The address domain was a single chain antibody (scFvαHA) derived from monoclonal antibody mAb26/9, which recognized an influenza virus haemaglutinin (HA) epitope DVPDYA (SEQ ID NO: 18) [18]. The enzyme domain was prethrombin (residues 315 to 622 of human prothrombin; accession no. AAC63054) - a zymogen of thrombin that could be activated using Factor Xa. Address and enzyme domains were connected with a 15 amino acid linker ($[GGGGS]_3$, SEQ ID NO: 19). When tested against a target containing DVPDYA (SEQ ID NO: 18) and a suboptimal thrombin cleavage site (*e.g.*, GGVR, SEQ ID NO: 20), the thrombin domain in the adzyme demonstrates accelerated cleavage because of the higher local concentration of peptide achieved through binding to DVPDYA (SEQ ID NO: 18) by the scFv domain (the address domain).

Both N-terminal and C-terminal fusions of adzymes are created with a variety of tags (myc, His_6 (SEQ ID NO: 10), V5). Different linker compositions and lengths are used. For example, the following constructs may be created: thrombin-tag-COOH; scFvαHA-tag-COOH; N-thrombin-linker-scFvαHA-tag-COOH; N-scFv αHA-linker-thrombin-tag-COOH; N-

scFvαHA-linker-thrombin-linker-scFvαHA-tag-COOH; or constructs with two thrombin units in tandem along with scFv anti-HA.

Prethrombin and the single chain antibody directed against the HA epitope are cloned individually into the HindIII and XhoI sites of the pSecTag2A vector from Invitrogen to generate proteins that will be secreted into the medium for subsequent biochemical characterization. Prethrombin is the inactive form that is activated by Factor Xa or ecarin. Prethrombin$(G_4S)_3$-scHA (SEQ ID NO: 19) and scHA$(G_4S)_3$-prethrombin (SEQ ID NO: 19) are assembled by overlap / recombinant PCR (using the oligos described in Table X below) and cloned into the pSecTag2A vector as HindIII and XhoI fragments. They will contain myc and His_6 (SEQ ID NO: 10) as tags at the C-terminus. The slash shows where the cleavage occurs in the signal peptide. The amino acid sequence for Prethrombin$(G_4S)_3$ scFvαHA (SEQ ID NO: 19) is:

METDTLLLWVLLLWVPGSTG/DAAQPARRAVRSLMTATSEYQTFFNPRTFGSGEADCG
LRPLFEKKSLEDKTERELLESYIDGRIVEGSDAEIGMSPWQVMLFRKSPQELLCGASLISD
RWVLTAAHCLLYPPWDKNFTENDLLVRIGKHSRTRYERNIEKISMLEKIYIHPRYNWRE
NLDRDIALMKLKKPVAFSDYIHPVCLPDRETAASLLQAGYKGRVTGWGNLKETWTAN
VGKGQPSVLQVVNLPIVERPVCKDSTRIRITDNMFCAGYKPDEGKRGDACEGDSGGPFV
MKSPFNNRWYQMGIVSWGEGCDRDGKYGFYTHVFRLKKWIQKVIDQFGEGGGGSGG
GGSGGGGSMEVQLLESGGDLVKPGGSLKLSCAASGFTFSTYGMSWVRQTPDKRLEWV
ATISNGGGYTYYPDSVKGRFTISRDNAKNTLYLQMSSLKSEDTAMYYCARRERYDENG
FAYWGRGTLVTVSAGGGGSGGGGSGGGGSDIVMSQSPSSLAVSVGEKITMSCKSSQSLF
NSGKQKNYLTWYQQKPGQSPKLLIYWASTRESGVPDRFTGSGSGTDFTLTISSVKAEDL
AVYYCQNDYSHPLTFGGGTKLEIKRADAAPTARGGPEQKLISEEDLNSAVDHHHHHH*(
SEQ ID NO: 21).

The amino acid sequence for scHA$(G_4S)_3$prethrombin (SEQ ID NO: 19) as made from pSecTag2 is:

METDTLLLWVLLLWVPGSTG/DAAQPARRAVRSLMEVQLLESGGDLVKPGGSLKLSCA
ASGFTFSTYGMSWVRQTPDKRLEWVATISNGGGYTYYPDSVKGRFTISRDNAKNTLYL
QMSSLKSEDTAMYYCARRERYDENGFAYWGRGTLVTVSAGGGGSGGGGSGGGGSDIV
MSQSPSSLAVSVGEKITMSCKSSQSLFNSGKQKNYLTWYQQKPGQSPKLLIYWASTRES

GVPDRFTGSGSGTDFTLTISSVKAEDLAVYYCQNDYSHPLTFGGGTKLEIKRADAAPTG
GGGSGGGGSGGGGSMTATSEYQTFFNPRTFGSGEADCGLRPLFEKKSLEDKTERELLES
YIDGRIVEGSDAEIGMSPWQVMLFRKSPQELLCGASLISDRWVLTAAHCLLYPPWDKNF
TENDLLVRIGKHSRTRYERNIEKISMLEKIYIHPRYNWRENLDRDIALMKLKKPVAFSDY
IHPVCLPDRETAASLLQAGYKGRVTGWGNLKETWTANVGKGQPSVLQVVNLPIVERPV
CKDSTRIRITDNMFCAGYKPDEGKRGDACEGDSGGPFVMKSPFNNRWYQMGIVSWGE
GCDRDGKYGFYTHVFRLKKWIQKVIDQFGEARGGPEQKLISEEDLNSAVDHHHHHH*
(SEQ ID NO: 22).

TABLE X

Oligo Name	Alternative name	Sequence (5' to 3')	SEQ ID NOS	Length	Tm	Purpose
B1	scHAfwdHindIII	CCCGGAAGCTTAatggaggtgcagctgttg	23	30	56	Fwd primer for amplifying ScHA for cloning into pSecTag2A using HindIII. A added after HindIII site to maintain reading frame.
B2	scHArevXhoI	acgcccCTCGAGCagttggtgcagcatcagc	24	31	56	Reverse primer for amplifying scHA for cloning into pSecTag2A using XhoI. C added prior to XhoI site to maintain reading frame.
B3	prethrombinfwdH3	CCCGGAAGCTTAATGaccgccaccagtgagtac	25	33	58	Fwd primer for amplifying prethrombin into pSecTag2A using HindIII. A added to keep frame after HindIII.
B4	prethrombinrevXhoI	ggcccCTCGAGCctctccaaactgatcaatg	26	31	56	Rev primer to clone prethrombin into XhoI site of pSecTag2A. C added to keep frame.
B5	G4ScHAfwd	tttggagagggaggcggtgggtctggtgggggcggtagtg gcggaggtgggagcatggaggtgcagctgttg	27	72	56	Forward primer to introduce (G4S)3 at 5' end of scHA.
B6	prethrombinG4Srev	cacctccatgctcccacctccgccactaccgcccccacca gacccaccgcctccctctccaaactgatcaatg	28	73	54	Reverse primer to introduce (G4S)3 tag at the 3' end of prethrombin.
B7	G4Sprethrombinfwd	gcaccaactggaggcggtgggtctggtgggggcggtagt ggcggaggtgggagcATGaccgccaccagtgagtac	29	75	58	Fwd primer to amplify prethrombin with (G4S)3 at 5' end to create overlap with ScHA.

B8	scHAG4Srev	ggtggcggtCATgctcccacctccgccactaccgccccc accagacccaccgcctccagttggtgcagcatcagc	30	75	56	Rev primer to amplify scHA with (G4S)3 at 3' end to create overlap with G4Sprethrombin.

Table X discloses the "G4S" and "(G4S)3" sequences as SEQ ID NOS 17 and 19, respectively.

Substrates tested include: S1, a high affinity epitope (DVPDYA, SEQ ID NO: 18) recognized by scFv αHA linked to the proteolytic target site (HAE-PT: NH_2-YPYDVPDYA-$(SGSGS)_4$-GGVR-p-nitroanilide, SEQ ID NO: 31); and S2, the proteolytic target alone (PT: NH_2-GGVR-p-nitroanilide (SEQ ID NO: 20)). Other synthetic peptide substrates were also made with variable binding and cleaving substrate sequences. The Thrombin cleavage sites were chosen based on the teachings of Backes *et al.* (2000) *Nature Biotechnology* 18:187-193. Alternate choices include Ile-Thr-Pro-Arg (SEQ ID NO: 32) as the best cleavage site and Ile-Thr-Leu-Arg (SEQ ID NO: 33) as a poor target.

Please delete the paragraph on page 144, lines 5-18 and replace it with the following paragraph:

Briefly, mammalian expression vector pSecTag2A (Cat. No.V90020; Invitrogen, Carlsbad, CA) was used as the backbone for all constructs. Upstream of the polylinker is a murine Ig κ-chain V-J2-C signal peptide, and downstream are myc and His_6 (SEQ ID NO: 10) tags, a TAA stop codon and a bovine growth hormone polyadenylation signal. Other notable features of the vector are a cytomegalovirus (CMV) promoter to drive expression of the inserted coding sequence and the selectable markers zeocin and ampicillin. cDNAs corresponding to individual components were generated by PCR and cloned directionally into the polylinker to maintain the reading frame using HindIII at the 5' end and XhoI at the 3' end. The address component (scFvαHA) was amplified from a plasmid template containing the coding sequence of scFvαHA (engeneOS, Waltham, MA); prethrombin was amplified from the full length human cDNA clone (ResGen; Cat. no. FL1001), and; the adzyme was created by overlap PCR designed to insert a 15 amino acid linker $(GGGGS)_3$ (SEQ ID NO: 19) between the N-terminal prethombin domain and the C-terminal address domain. All constructs were sequence confirmed.

Please delete the paragraph on page 144, line 33 to page 145, line 8 and replace it with the following paragraph:

As shown in Figure 5, the model adzyme prethrombin-$(GGGGS)_3$-scFvαHA (SEQ ID NO: 19) was expressed transiently in 293T cells and conditioned media harvested on day 7. The material was processed and purified as described above. Samples representing equivalent portions of each fraction were loaded onto 4-20% polyacrylamide gels and electrophoresed in Tris-glycine-SDS buffer (Novex). Panel A. Western blot—following electrophoresis the gel was electroblotted to nitrocellulose membranes which were stained with an anti-myc antibody (Invitrogen, Carlsbad, CA). Lane (1) Load; (2) Flow through; (3) Wash 1; (4) Wash 3; (5) Elution 1; (6) Elution 2; (7) Elution 3; (8) Resin boiled in sample loading buffer; (9) Cruz mol. weight marker (Santa Cruz Biotechnology, Santa Cruz, CA). Panel B: Silver-stained gel. Lane (1) starting material; (2) Flow through; (3) Wash 1; (4) Wash 3; (5) molecular weight standard SeeBlue Plus 2; (6) Elution 1; (7) Elution 2; (8) Elution 3; (9) Resin boiled in sample loading buffer; (10) molecular weight standard SeeBlue Plus 2.

Please delete the paragraph on page 146, lines 10-20 and replace it with the following paragraph:

Binding to a target epitope. This experiment assessed the binding characteristics of the adzyme address domain. Applicants assessed binding activity of various components using biotinylated peptides in a sandwich ELISA format. Purified components were dialyzed against PBS, captured on plates coated with anti-myc antibody (mAb 9E10; Sigma), then analyzed by ELISA for binding to biotinylated target peptide (NH_2-YPYDVPDYAGSGDYKAFD, SEQ ID NO: 34), which contained the high affinity epitope (underline). Bound peptides were quantified using a streptavidin-horseradish peroxidase detection system (Quantablue; Pierce, Rockford, IL). The address domain alone and both the activated and zymogen forms of the adzyme bound comparable levels of the peptide per mole. However the enzyme domain alone failed to bind measurable amounts of the peptide, as expected.

Please delete the paragraph on page 147, lines 22-32 and replace it with the following paragraph:

Normalization of proteolytic activity. Applicants needed to quantify the enzymatic activity of the model thrombin-$(GGGGS)_3$scFvαHA (SEQ ID NO: 19) adzyme with reference to the standard human thrombin. The commercially available tripeptide tosyl-GPR-pNA (Sigma), which lacked the high affinity HA binding site was used as substrate. Cleavage of the peptide bond following the Arg residue releases released the chromophore p-nitroaniline (pNA) which is visible at 405 nm. Applicants determined the relative proteolytic activity, in units of thrombin activity per ml, of adzyme components before and after activation with Factor Xa. Factor Xa has no activity on the commercial substrate. Data from one such experiment are shown below in Fig. 7. This allowed normalization based on enzymatic activity of the adzyme preparation and comparison of equivalent activities for adzyme and native commercial thrombin against substrate S1 and S2.

Please delete the paragraphs on page 148, line 3 to page 149, line 12 and replace them with the following paragraphs:

Results of this experiment showed that this model thrombin-$(GGGGS)_3$scFvαHA (SEQ ID NO: 19) adzyme preparation: 1) had no detectable activity prior to activation and; 2) could be normalized against a standard thrombin preparation—in this case 5 µl/ml of the activated model adzyme was equivalent to 3.3 nM (0.1 NIH U/ml) of thrombin. Active site titration of activated samples with D-FPR-CMK provided independent verification of the normalization. Hence, the proteolytic activity for adzyme preparations were normalized relative to the thrombin standard.

In summary, these control experiments have shown that: 1) the address domain-mediated binding to the high affinity epitope and linkage of an enzyme domain did not interfere with binding activity; 2) the activated model thrombin-$(GGGGS)_3$scFvαHA (SEQ ID NO: 19) adzyme had a K_m value comparable to thrombin for a standard thrombin substrate; 3) thrombin had equivalent specificity for substrates S1 and S2; 4) activation using Factor Xa was required to obtain detectable proteolytic activity; and 5) Applicants were able to normalize the proteolytic

activities of adzyme preparations relative to a commercial thrombin standard. This series of control experiments have provided the basis for testing and comparing the adzyme and isolated components on substrates that contained or lacked a high affinity epitope for the address domain.

2.3. Test of Adzyme Function.

Applicants have designed an adzyme, thrombin-$(GGGGS)_3$scFvαA (SEQ ID NO: 19), comprising a prethrombin enzyme domain linked by a 15 amino acid polypeptide to a single chain antibody to the HA epitope as the address domain. Thrombin does not bind or cleave the HA epitope but binds its targeted substrate site GGVR (SEQ ID NO: 20), whether in the context of S1 or S2, with the same affinity. The activated thrombin component of the thrombin-scFvαHA adzyme also binds the GGVR (SEQ ID NO: 20) of S1 with the same affinity; however the adzyme concept predicts that thrombin coupled to the anti-HA antibody will bind to substrates containing the HA epitope with the typical higher affinities of antibodies and may affect the adzyme reaction rate. It is predicted that the adzyme could have heightened enzymatic activity compared to thrombin.

In the reaction velocity experiments using the substrates S1 and S2 with either thrombin or thrombin-(GGGGS)3scFvαHA (SEQ ID NO: 19) adzyme; it is predicted that: 1) the address domain alone (A) would be inactive (-) on both substrates; 2) the enzyme alone (B) and the adzyme (D) would have equivalent (+) proteolytic activity on substrate S2, the thrombin cleavage site alone; 3) the adzyme would be more active (+++) against substrate S1 (S1 has both the high affinity epitope and the thrombin cleavage site) than against substrate S2 or the enzyme alone against either substrate (+); and 5) a stoichiometric mixture (C) of the unlinked address domain and enzyme domain would be equivalent to the enzyme domain alone on both substrates (+) (see Table 1) and less than the adzyme.

Table 1: Model thrombin-(GGGGS)3scFvαHA (SEQ ID NO: 19) adzyme and components tested against linear peptide substrates

	Substrate	
Test component	S1: HAE-PT	S2:PT

A	scFvαHA	-	-
B	Thrombin	+	+
C	A + B	+	+
D	Thrombin-$(GGGGS)_3$scFvαHA (SEQ ID NO: 19)	+++	+

Please delete the paragraph on page 149, line 24 to page 150, line 2 and replace it with the following paragraph:

Equivalent activities of the activated thrombin-$(GGGGS)_3$scFvαHA (SEQ ID NO: 19) adzyme and activated commercial thrombin, as determined with the toysl-GPR-pNA substrate and hence normalized, were tested against S1 and S2. As shown in Figure 8, the reaction rate for both the adzyme and thrombin are the same on the S2 substrate which contains just the thrombin cleavage site as expected, since both the adzyme preparations had been normalized to thrombin. However, as predicted, the model adzyme showed increased activity towards substrate S1 which contained a high affinity epitope in addition to the thrombin cleavage site. There is a 2X increase in reaction rate. The presence of this high affinity epitope on the substrate did not alter the activity of the thrombin alone. In the absence of activation the adzyme did not show detectable proteolytic activity. Thus the enhanced activity of thrombin-$(GGGGS)_3$scFvαHA (SEQ ID NO: 19) adzyme is driven by the presence of an address domain that directed the enzyme activity to the substrate through binding a high affinity epitope.

Please delete the paragraph on page 150, line 33 to page 151, line 6 and replace it with the following paragraph:

Specifically, the enzymes are: cationic trypsin and MMP7; the addresses are: Sp55, Sp55_2.6, and scFv; the linkers are: linkers with 0, 10, 20, 30, 40, or 50 amino acids

(corresponding to repeating units of GGGGS (SEQ ID NO: 17)), FcIgG1 (knob mutation), FcIgG1 (hole mutation), FcIgG2 (knob mutation), FcIgG2 (hole mutation), FcIgG3 (knob mutation), FcIgG3 (hole mutation), FcIgG2-$(G_4S)_2$ (SEQ ID NO: 35) hole mutation, FcIgG2-$(G_4S)_4$ (SEQ ID NO: 36) hole mutation, FcIgG2-$(G_4S)_3$ (SEQ ID NO: 19) hole mutation, FcIgG2-$(G_4S)_4$ (SEQ ID NO: 36) hole mutation. The knob and hole mutations refer to the paired mutations (S354C:T366'W/Y349C:T366S:L368'A:Y407'V)in CH3 domains that had been identified as giving rise to predominantly heterodimeric bispecific antibodies (Merchant et al. Nature Biotechnology, 1998, 16, p. 677-681).

Please delete the paragraphs on page 155, lines 10-33 and replace them with the following paragraphs:

c. Selection of the linkers A significant function of a linker is to connect a catalytic domain and an address domain in a fusion protein to yield cooperative function. The linker lengths can be experimentally investigated. Applicants found that a triple-repeat (or "3-repeat") of the flexible pentapeptide GGGGS (SEQ ID NO: 19) enabled a functional linkage of the enzyme and address domains. This linker can range in length from 23.60 Å in α-helical conformation to 50.72 Å as an extended chain. The initial adzymes have been built with 0 amino acids as linker (to minimize intramolecular digestion, 3 amino acids (AAA) and 20 amino acids (4 repeats of G_4S) (SEQ ID NO: 36). Additional linker lengths under construction are 2 repeats of G_4S (10 amino acids) (SEQ ID NO: 35), 6 repeats of G_4S (30 amino acids) (SEQ ID NO: 37), 8 repeats of G_4S (40 amino acids) (SEQ ID NO: 38) and 10 repeats of G_4S (50 amino acids) (SEQ ID NO: 39).

	Extended form	α-helical form
$(GGGGS)_2$ (SEQ ID NO: 35)	32.02 Å	15.96 Å
$(GGGGS)_4$ (SEQ ID NO: 36)	64.04 Å	31.92 Å
$(GGGGS)_6$ (SEQ ID NO: 37)	96.06 Å	47.88 Å
$(GGGGS)_8$ (SEQ ID NO: 38)	128.08 Å	63.84 Å

$(GGGGS)_{10}$ (SEQ ID NO: 39)	160.1 Å	79.8 Å

d. Adzyme Structures There are currently no reports in the literature for heterologous expression of trypsin in mammalian cells. Thus, it might be prudent to express the zymogen form that could be activated by enterokinase. Trypsinogen was thus cloned to be in frame with the leader sequence and N-terminal to the linker and address domain and in frame with the tandem myc-His_6 (SEQ ID NO: 10) tags at the C-terminus.

N-murine Igκ leader sequence-trypsinogen-0aa-sp55-myc-His6 tgn-0-sp55 (SEQ ID NO: 10)

N-murine Igκ leader sequence-trypsinogen-AAA-sp55-myc-His6 tgn-3-sp55 (SEQ ID NO: 10)

N-murine Igκ leader sequence-$(G_4S)_4$-trypsinogen-20aa-sp55-myc-His6 tgn-20-sp55 (SEQ ID NOS 36 and 10, respectively)

Please delete the paragraph on page 156, lines 31-35 and replace it with the following paragraph:

Recombinant adzymes may be generated using the pSecTag2A vector system or any other equivalently functional system for transient expression in mammalian cells. The adzymes can be purified, for example, from conditioned media by binding the His_6 (SEQ ID NO: 10) tags to a nickel resin. Additional technical details are described in example section 3.1.a., above. All adzyme constructs generated in this section have been sequence confirmed.

Please delete the paragraphs on page 157, line 10 to page 158, line 21 and replace them with the following paragraphs:

Also in this particular example, the address domain used is sp55, although other address domains such as scFV anti-TNFα antibody may also be used (both selected from a set of 18 potential candidates). All of these constructs when completed are verified by DNA sequencing.

The amino acid sequence of trypsinogen (tgn) is:

METDTLLLWVLLLWVPGSTG↓DIAPFDDDDKIVGGYNCEENSVPYQVSLNSGYHFCGGS
LINEQWVVSAGHCYKSRIQVRLGEHNIEVLEGNEQFINAAKIIRHPQYDRKTLNNDIMLI
KLSSRAVINARVSTISLPTAPPATGTKCLISGWGNTASSGADYPDELQCLDAPVLSQAKC
EASYPGKITSNMFCVGFLEGGKDSCQGDSGGPVVCNGQLQGVVSWGDGCAQKNKPGV
YTKVYNYVKWIKNTIAANSTRGGPEQKLISEEDLNSAVDHHHHHH* (SEQ ID NO: 40)

The amino acid sequence of trypsinogen-0aa-sp55 (tgn-0-sp55) as expressed from pSecTag2A is:

METDTLLLWVLLLWVPGSTG↓DIAPFDDDDKIVGGYNCEENSVPYQVSLNSGYHFCGGS
LINEQWVVSAGHCYKSRIQVRLGEHNIEVLEGNEQFINAAKIIRHPQYDRKTLNNDIMLI
KLSSRAVINARVSTISLPTAPPATGTKCLISGWGNTASSGADYPDELQCLDAPVLSQAKC
EASYPGKITSNMFCVGFLEGGKDSCQGDSGGPVVCNGQLQGVVSWGDGCAQKNKPGV
YTKVYNYVKWIKNTIAANSLVPHLGDREKRDSVCPQGKYIHPQNNSICCTKCHKGTYL
YNDCPGPGQDTDCRECESGSFTASENHLRHCLSCSKCRKEMGQVEISSCTVDRDTVCGC
RKNQYRHYWSENLFQCFNCSLCLNGTVHLSCQEKQNTVCTCHAGFFLRENECVSCSNC
KKSLECTKLCLPQIENVKGTEDSGTTRGGPEQKLISEEDLNSAVDHHHHHH* (SEQ ID NO: 41)

The amino acid sequence of trypsinogen-3aa-sp55 (tgn-3-sp55) as expressed from pSecTag2A is:

METDTLLLWVLLLWVPGSTG↓DIAPFDDDDKIVGGYNCEENSVPYQVSLNSGYHFCGGS
LINEQWVVSAGHCYKSRIQVRLGEHNIEVLEGNEQFINAAKIIRHPQYDRKTLNNDIMLI
KLSSRAVINARVSTISLPTAPPATGTKCLISGWGNTASSGADYPDELQCLDAPVLSQAKC
EASYPGKITSNMFCVGFLEGGKDSCQGDSGGPVVCNGQLQGVVSWGDGCAQKNKPGV
YTKVYNYVKWIKNTIAANSAAALVPHLGDREKRDSVCPQGKYIHPQNNSICCTKCHKG
TYLYNDCPGPGQDTDCRECESGSFTASENHLRHCLSCSKCRKEMGQVEISSCTVDRDTV
CGCRKNQYRHYWSENLFQCFNCSLCLNGTVHLSCQEKQNTVCTCHAGFFLRENECVSC
SNCKKSLECTKLCLPQIENVKGTEDSGTTRGGPEQKLISEEDLNSAVDHHHHHH* (SEQ ID NO: 42)

The amino acid sequence of trypsinogen-20aa-sp55 (tgn-20-sp55) as expressed from pSecTag2A is:

METDTLLLWVLLLWVPGSTG↓DIAPFDDDDKIVGGYNCEENSVPYQVSLNSGYHFCGGS
LINEQWVVSAGHCYKSRIQVRLGEHNIEVLEGNEQFINAAKIIRHPQYDRKTLNNDIMLI
KLSSRAVINARVSTISLPTAPPATGTKCLISGWGNTASSGADYPDELQCLDAPVLSQAKC
EASYPGKITSNMFCVGFLEGGKDSCQGDSGGPVVCNGQLQGVVSWGDGCAQKNKPGV
YTKVYNYVKWIKNTIAANSAAAGGGGSGGGGSGGGGSGGGGSRLVPHLGDREKRDSV
CPQGKYIHPQNNSICCTKCHKGTYLYNDCPGPGQDTDCRECESGSFTASENHLRHCLSC
SKCRKEMGQVEISSCTVDRDTVCGCRKNQYRHYWSENLFQCFNCSLCLNGTVHLSCQE
KQNTVCTCHAGFFLRENECVSCSNCKKSLECTKLCLPQIENVKGTEDSGTTRGGPEQKLI
SEEDLNSAVDHHHHHH* (SEQ ID NO: 43)

In addition, sp55 was also cloned into pSecTag in similar fashion. The amino acid sequence of sp55 as expressed from pSecTag2A is:

METDTLLLWVLLLWVPGSTG↓DAAQPARRAVRSLVPHLGDREKRDSVCPQGKYIHPQN
NSICCTKCHKGTYLYNDCPGPGQDTDCRECESGSFTASENHLRHCLSCSKCRKEMGQVE
ISSCTVDRDTVCGCRKNQYRHYWSENLFQCFNCSLCLNGTVHLSCQEKQNTVCTCHAG
FFLRENECVSCSNCKKSLECTKLCLPQIENVKGTEDSGTTRGGPEQKLISEEDLNSAVDH
HHHHH* (SEQ ID NO: 44)

Please delete the paragraphs on page 159, line 6 to page 160, line 7 and replace them with the following paragraphs:

c. adzyme Purification In one embodiment, His_6-nickel (SEQ ID NO: 10) methodology is the preferred method of purification. This method is rapid, simple and available in either column format for large batches or in a 96 well format for parallel assay testing. However, many other alternative methods of purification can be used. For example, one option could be benzamidine sepharose column chromatography (Pharmacia, NJ), which incorporates a protease inhibitor into the resin. Standard characterization of purified proteins will include Western analysis with anti-myc antibodies and silver-stained gels to assess purity and recovery of the adzyme preparations. The produced adzymes may be further analyzed to quantify binding and proteolytic activities.

d. Recombinant protein determination. In one embodiment, adzymes are constructed with a carboxy terminal tandem myc-His_6 (SEQ ID NO: 10) tags. An ELISA method is developed to detect the c-myc tag for quantitating recombinant proteins bound to Ni-NTA on surfaces. This helps to normalize the amount of adzyme used in any biochemical analyses and bioassays.

The following method can be used to quantify heterologously expressed proteins containing tandem myc and His_6 (SEQ ID NO: 10) tags using a sandwich ELISA approach. In summary, diluted conditioned medium containing recombinant proteins are incubated in wells of Ni-NTA coated HisSorb microtiter plates (catalog no. 35061 Qiagen, Valencia, CA) and then reacted with anti-myc-HRP (catalog no. R951-25, Invitrogen, Carlsbad, CA). Bound recombinant material is then detected by incubation with a chromogenic substrate. A standard curve was established in parallel with purified recombinant sp55 (independently quantified using a commercially available ELISA (catalog no. QIA98, Oncogene Research Products, Madison, WI) containing tandem myc His_6 (SEQ ID NO: 10) tag allowing quantification of captured material. Conditioned media from mock transfected cells served as a negative control.

In brief, conditioned media from transfections was diluted directly into assay buffer (0.5 % BSA Fraction V, 0.05 % Tween-20 in 1 X PBS pH 7.4) to a final volume of 100 µL / well. Known amounts of the standard, sp55, was serially diluted in similar fashion in assay buffer. Binding of the His_6 (SEQ ID NO: 10) tag of the recombinant proteins to the Ni-NTA surface was allowed to proceed at room temperature for half an hour with slow shaking. Anti-myc-HRP was then added to all wells at a final dilution of 1:1500 such that the final volume in the wells was 150 µL. Binding was allowed to proceed for two hours at room temperature with slow shaking. Following the binding of anti-myc to the His_6-captured (SEQ ID NO: 10) proteins, the wells were washed 6 times with wash buffer (PBS containing 0.05% Tween 20) and blotted dry. Then, the chromogenic substrate TMB (Sigma Catalog #T-4444) was added to each of the wells to a final volume of 100 µL. The increase in absorbance at 370 nm was monitored by a microtiter plate UV/VIS reader (Molecular Devices SPECTRAmax 384 Plus). All samples are assayed in duplicate.

Please delete the paragraphs on page 162, line 19 to page 163, line 9 and replace them with the following paragraphs:

Applicants have developed a method for carrying out on-plate capture, activation and proteolytic assays for recombinantly produced enzymes or adzymes containing a His_6 (SEQ ID NO: 10) tag. In summary, diluted conditioned medium containing recombinant proteins are incubated in wells of Ni-NTA coated HisSorb microtiter plates, then treated with enterokinase and presented with suitable peptide substrates. The peptide substrate used in the current example is tosyl-GPR-AMC (Catalog no. 444228, Sigma, St. Louis, MO) which has been described previously. Proteolysis of the peptide bond between the Arg residue in the substrate and the AMC leads to the release of free fluorescent AMC (excitation 383nm, emission 455 nm). Inclusion of conditioned media from sp55 or vector transfections provide important negative controls for the levels of adventitious protease expression in transfected cells and substrate background and hydrolysis under assay conditions.

In brief, conditioned medium containing recombinant proteins was diluted directly into assay buffer (0.5 % BSA Fraction V, 0.05 % Tween-20 in 1 X PBS pH 7.4) to a final volume of 100 µL / well. Typically, 5-25% of conditioned medium per well yielded good linear response. Binding of the His_6 (SEQ ID NO: 10) tag of the recombinant proteins to the Ni-NTA surface was allowed to proceed at room temperature for two hours with slow shaking. Following the binding of anti-myc to the His_6-captured (SEQ ID NO: 10) proteins, the wells were washed 6 times with wash buffer (PBS containing 0.05% Tween 20 or PBST, 200 µL per wash) and blotted dry. This step also accomplishes the removal of benzamidine which would otherwise interfere with subsequent steps in the assay. Activation of zymogen is achieved by the addition of 1 U of enterokinase (EK, Catalog no. 69066, Novagen, Madison, WI) in a final volume of 100 uL of PBST. Activation was carried out for 1 hour at 37°C. A parallel set of samples received no enterokinase but underwent similar incubation. Finally, the wells were washed 6 times with PBST prior to the addition of trypsin digestion buffer (100 mM Tris pH 8, 5 mM $CaCl_2$) containing 10 µM tosyl-GPR-AMC. Proteolytic activity was followed by monitoring the fluorescence at 455 nm following excitation at 383 nm using a Gemini EM microplate spectrofluorometer (Molecular Devices, CA).

Please delete the paragraph on page 163, lines 32-33 and replace it with the following paragraph:

The assay for MMP7 proteolytic activity may use a fluorogenic substrate (dinitrophenyl-RPLALWRS (SEQ ID NO: 45); Calbiochem Cat. No. 444228).